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Sulfation-dependent Recognition of High Endothelial Venules (HEV)-Ligands by L-Selectin and MECA 79, an Adhesion-blocking Monoclonal Antibody

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Summary

L-selectin is a lectin-like receptor that mediates the attachment of lymphocytes to high endothelial venules (HEV) of lymph nodes during the process of lymphocyte recirculation. Two sulfated, mucin-like glycoproteins known as Sgp50/GlyCAM-1 and Sgp90/CD34 have previously been identified as HEV-associated ligands for L-selectin. These proteins were originally detected with an L-selectin/Ig chimera called LEC-IgG. GlyCAM-1 and CD34 are also recognized by an anti-peripheral node addressin (PNAd) mAb called MECA 79, which blocks L-selectin–dependent adhesion and selectively stains lymph node HEV. The present study compares the requirements for the binding of MECA 79 and LEC-IgG to HEV-ligands. Whereas desialylation of GlyCAM-1 and CD34 drastically reduced binding to LEC-IgG, this treatment enhanced the binding of GlyCAM-1 to MECA 79. In contrast, the binding of both MECA 79 and LEC-IgG to GlyCAM-1 and CD34 was greatly decreased when the sulfation of these ligands was reduced with chlorate, a metabolic inhibitor of sulfation. Because MECA 79 stains HEV-like vessels at various sites of inflammation, recognition by L-selectin of ligands outside of secondary lymphoid organs may depend on sulfation. In addition to their reactivity with GlyCAM-1 and CD34, both MECA 79 and LEC-IgG recognize an independent molecule of ~200 kD in a sulfate-dependent manner. Thus, this molecule, which we designate Sgp200, is an additional ligand for L-selectin.

L-selectin, originally identified on mouse lymphocytes by the mAb MEL-14 (1, 2), mediates the attachment of lymphocytes to high endothelial venules (HEV)[1] of lymph nodes during the process of lymphocyte recirculation. The widespread distribution of L-selectin on all leukocyte classes underlies its more general participation in leukocyte–endothelial interactions during a variety of inflammatory reactions (3–5). A potential role for L-selectin in the pathophysiologic targeting of leukocytes to certain extravascular sites has also been proposed (6). As a member of the selectin family of cell–cell adhesion proteins, L-selectin contains an extracellular calcium-type lectin domain (7), which dictates its adhesive function (8–11). The preferential involvement of L-selectin in lymphocyte trafficking to lymph nodes, as opposed to mucosal lymphoid organs such as Peyer's patches (12), originally motivated the search for cognate HEV-ligands that are distributed in an organ-specific manner. Two different approaches have been employed to identity such ligands. One approach has used an immunoglobulin chimera of L-selectin, designated mouse L-selectin/Ig chimeric receptor or LEC-IgG, as a soluble receptor analogue (13). LEC-IgG selectively stains HEV in lymph nodes sections and blocks lymphocyte attachment to lymph node HEV (13). Employing this chimera as an affinity reagent, Imai et al. (14) precipitated two glycoproteins of ~50 and ~90 kD from lymph nodes that had been metabolically labeled with [^{35}S]SO_4. These two ligands, originally designated as sulfated glycoprotein (Sgp)50 and Sgp90, were subsequently identified at the molecular level as GlyCAM-1 (15) and a HEV-specific glycoform of CD34 (16). GlyCAM-1 is a secreted glycoprotein that may also have a peripheral membrane association with the apical plasma membrane of HEV endothelial cells (15, 17), whereas CD34 is a transmembrane glycoprotein found on endothelial cells and hematopoietic stem cells (18). Both GlyCAM-1 and Sgp90/CD34 are mucin-like glycoproteins that bear sulfated, sialylated, and fucosylated O-linked carbohydrate chains (14). For both ligands, sialylation is essential for recognition by

[1] *Abbreviations used in this paper:* AAA, *Aleuria aurantia* agglutinin; HEV, high endothelial venule; LEC-IgG, mouse L-selectin/Ig chimeric receptor; LFA, *Limax flavus* agglutinin; PLN, peripheral lymph node; PNAd, peripheral lymph node vascular addressin; Sgp, sulfated glycoprotein.

L-selectin (14, 19), consistent with the finding that in vivo or in vitro desialylation of HEV markedly reduces lymphocyte attachment (20, 21). Although fucosylation has not been directly demonstrated as an essential modification of these ligands, its importance is strongly suspected based on the observation that sialyl Lewis X, although a very weak inhibitor/ligand (19, 22, 23) for L-selectin, is superior to 3′ sialyl lactose (neu5Acα2 → 3Galβ1 → 4Glc) (19), which differs only in its lack of fucose. Also, an essential role for fucose has been established for the myeloid ligands of both P- and E-selectin (24), which possess calcium-type lectin domains that are highly homologous to that of L-selectin (8, 9). Finally, HEV express binding sites for E-selectin (25), CSLEX mAb (26), and HECA 452 mAb (22), whose carbohydrate epitopes all require fucose.

Sulfation has long been suspected to be an important modification of lymph node HEV-ligands, stemming from the original observation that lymph node HEV are sites of avid incorporation of [^{35}S]SO_4 into macromolecules (27). Additional interest in the sulfation of HEV-ligands has derived from the knowledge that several sulfated glycoconjugates, although completely lacking in sialylation, exhibit specific ligand activity for L-selectin (28–33). A direct role for the sulfation of GlyCAM-1 was first demonstrated using chlorate, a highly selective metabolic inhibitor of sulfation (34). Undersulfated GlyCAM-1, produced in organ culture of lymph nodes with chlorate present, shows dramatically reduced binding to L-selectin. Direct analysis of the GlyCAM-1 carbohydrates has been undertaken with the objective of defining a minimal recognition determinant that accommodates the requirements for sialylation, fucosylation, and sulfation (35–37).

An independent approach to the identification of HEV-ligands for L-selectin has relied on the use of a mAb strategy. Streeter et al. (38) described an anti-peripheral lymph node (PLN) addressin mAb (i.e., MECA 79) that stains lymph node HEV and blocks L-selectin–dependent lymphocyte attachment both in vitro and in vivo. In contrast, the luminal staining of HEV in Peyer's patches is weak, consistent with the lesser role of L-selectin at this site (38, 39). The function-blocking activity of MECA 79 has recently been extended to HEV in human lymph nodes and tonsils (40). By Western blotting or radioiodination of immunoprecipitated material, MECA 79 reacts with a complex set of proteins in both human tonsils and mouse lymph nodes (41, 42). It is important to note that GlyCAM-1 and CD34, as isolated with LEC-IgG from mouse lymph nodes, carry the epitope for MECA 79 (14). Moreover, the set of MECA 79-precipitated bands (detected by radioiodination) from lymph nodes of young mice includes components at ~50 and ~90 kD as well as five additional species (42) (see Discussion). The isolated complex of MECA 79–reactive proteins (PLN addressin [PNAd]) is able to support specific lymphocyte attachment via L-selectin in a sialidase-sensitive manner (41). The occurrence of the MECA 79 epitope on multiple components has suggested that it might represent a carbohydrate posttranslational modification closely associated with the determinants that confer binding activity for L-selectin (41). Although the MECA 79–defined modification has clearcut functional significance, as adduced from the function-blocking activity of the antibody and the adhesion-promoting activity of the PNAd complex, the biochemical nature of the epitope has heretofore not been examined.

In this study, we show that both L-selectin and MECA 79 mAb recognize the same set of [^{3}H]Gal-labeled and [^{35}S]SO_4-labeled glycoproteins from lymph nodes. Furthermore, we show that as for L-selectin, recognition of these HEV-ligands by MECA 79 is dependent on sulfation.

Materials and Methods

Materials. LEC-IgG was provided by Dr. L. Lasky and Dr. S. Watson (Genentech Inc., South San Francisco, CA) and immobilized on protein A-Sepharose 4B (Zymed Laboratories, Inc., South San Francisco, CA) at 10 mg/ml of gel. The rabbit polyclonal antiserum CAM02 against an internal peptide (peptide 2) of GlyCAM-1 has been described (15). Rabbit antiserum directed against recombinant murine CD34 (16) was a gift of Dr. L. Lasky and Dr. S. Baumhueter. Rabbit antibodies and preimmune serum were covalently cross-linked with dimethylpimelimidate (43) to protein A–Sepharose after saturating the protein A with serum. MECA 79 mAb (rat IgM) and a control rat IgM mAb (OZ42), described previously (38), were immobilized to a substitution level of 2 mg/ml gel on anti–rat Ig Sepharose (Sigma Chemical Co., St. Louis, MO). CAM02-peptide (CKEPSIFREELISKD) was synthesized by Dr. C. Glabe of the University of California (Irvine, CA). *Limax flavus* agglutinin was purchased from Calbiochem-Novabiochem (San Diego, CA). Recombinant *Aleuria aurantia* agglutinin (AAA) was the gift of Dr. A. Kobata and Dr. T. Endo (University of Tokyo, Tokyo, Japan). Both lectins were immobilized on CNBr-activated Sepharose 4B (Sigma Chemical Co.) at a level of 2 mg lectin/ml gel (44).

Metabolic Labeling of Murine Lymph Nodes. Murine axillary, brachial, cervical, and mesenteric lymph nodes from five ICR mice were split into two equal parts and labeled in parallel in organ culture (0.5 ml RPMI 1640, 4 h, 37°C), each containing 0.5 mCi/ml D-[6-^{3}H]galactose (Du Pont-NEN, Boston, MA). In one of the two cultures, sulfate content was depleted to 1/10 of normal and 10 mM sodium chlorate was added as an inhibitor of sulfation. For labeling with [^{35}S]SO_4, lymph nodes from five mice were labeled in organ culture (4 h, 37°C) in 1 ml RPMI 1640 with sulfate depleted to 1/10 normal and $Na_2{}^{35}SO_4$ (ICN, Irvine, CA) added at 1 mCi/ml.

Immunoprecipitations. To generate cell lysates, labeled tissues from control or chlorate-treated cultures were washed with 1 ml Dulbecco's PBS and then homogenized in 1 ml of PBS with 2% Triton X-100 supplemented with leupeptin (10 μg/ml), pepstatin A (10 μg/ml), aprotinin (0.28 U/ml trypsin inhibitor), benzamidine (0.75 mM), and PMSF (1 mM). Homogenates were centrifuged (5 min, 10,000 *g*) and supernatants precleared by 18 h incubation with 50 μl preimmune Ig-agarose. The supernatants were then immunoprecipitated sequentially (4 h/immunoprecipitation) with 10 μl of CD34 Ab–agarose followed by 20 μl of CAMO2 Ab–agarose. The immunoprecipitates were washed 5× with 1.5 ml of PBS containing 0.25% Triton X-100 and then boiled (5 min) in 0.4 ml of this buffer. For reprecipitation, each antigen preparation was split into four 100 μl aliquots. Three of the aliquots were incubated in parallel (4 h) with 2.5 μl LEC-IgG matrix (+ 2 mM $CaCl_2$), 5 μl MECA 79–agarose, or 5 μl *Limax flavus* agglu-

tinin–agarose. The fourth aliquot was boiled after addition of 5 μl 3× concentrated reducing SDS-sample buffer. Matrices were washed 4× with 0.5 ml of PBS containing 0.25% Triton X-100 and boiled in reducing SDS-sample buffer (150 μl final) for SDS-PAGE.

Lysates were also directly precipitated with LEC-IgG or MECA 79. Lysates prepared as above were precleared with 50 μl protein A–agarose and then precipitated with 10 μl of LEC-IgG matrix or alternatively were precleared with 10 μl OZ42–agarose plus 40 μl protein A–agarose and then precipitated with 10 μl of MECA 79–agarose. Precipitates were washed 5× with 1.5 ml of PBS 0.25% Triton X-100. The LEC-IgG matrix was eluted with 100 μl Tris-buffered saline (TBS; 10 mM Tris-Cl, 150 mM NaCl, pH 7.4) containing 5 mM EDTA, whereas the MECA 79 matrix was eluted by boiling in 100 μl of reducing SDS-sample buffer.

Exoglycosidase Digestions of GlyCAM-1. Conditioned medium was collected from the organ cultures above (cultured in the presence or absence of chlorate), and clarified by brief centrifugation (10,000 *g*, 5 min). After preclearing with 50 μl protein A–Sepharose, the conditioned medium was immunoprecipitated with 25 μl CAMO2 Ab–agarose. The beads were washed 5× with 1.5 ml of TBS and eluted with 1 mg/ml of CAMO2-peptide in TBS (200 μl). 10 μl aliquots of the eluate were reacted with 25 mU of *Arthrobacter ureafaciens* sialidase (Calbiochem-Novabiochem) alone or with the sialidase combined with 5 μU of *Streptomyces sp142* α(1→3/4) fucosidase (Takara Shuzo Co., Berkeley, CA) in 50 mM Na-cacodylate (pH 6.0), 0.05% BSA, 0.05% NaN_3, and 0.125% Triton X-100 (final volume 50 μl) for 48 h at 37°C. A control sample was incubated in parallel (48 h, 37°C) in buffer only. Each of the digests and the control sample were then split into four equal (12.5 μl) parts, of which three were diluted to 300 μl with PBS containing 0.1% BSA, 0.25% Triton X-100, and 0.02% NaN_3, while the fourth was boiled in reducing SDS-sample buffer (100 μl final). The three aliquots were then reprecipitated with 10 μl of either MECA 79-agarose, *Limax flavus* agglutinin–agarose, or AAA–agarose. Matrices were washed 4× with 0.5 ml of PBS containing 0.25% Triton X-100 and boiled in reducing SDS-sample buffer (100 μl final) for SDS-PAGE.

SDS-PAGE. SDS-PAGE (10% polyacrylamide gels) was carried out as described by Laemmli (45).

Results

We first examined the possible contribution of sialylation and fucosylation to the MECA 79 epitope on GlyCAM-1. GlyCAM-1 was metabolically labeled with [^{3}H]galactose in lymph node organ cultures and was isolated from conditioned medium with CAM02, a polyclonal antibody directed to an internal peptide sequence of GlyCAM-1 (15). To remove sialic acid, the isolated glycoprotein was treated with a broad spectrum sialidase from *A. ureafaciens*. As shown in Fig. 1 and Table 1, desialylation was effective, since the treated glycoprotein completely lost its reactivity with *Limax* agglutinin, a lectin with broad specificity for sialic acid (44). Desialylated GlyCAM-1 retained its ability to bind to MECA 79 mAb, as seen by visual inspection of the gel (Fig. 1). In fact, quantitation by densitometry revealed significantly increased (~70%) binding of MECA 79 to desialylated GlyCAM-1 as compared with normal GlyCAM-1.

To remove fucose, isolated GlyCAM-1 was treated with a combination of *Arthrobacter* sialidase and *Streptomyces* α(1→3/4) fucosidase. We previously demonstrated that this fucosidase removes ~70% of total fucose from desialylated GlyCAM-1 but is inactive on sialylated GlyCAM-1 (36). We confirmed that sialidase/fucosidase-treatment of [^{3}H]galactose-labeled GlyCAM-1 removed a substantial fraction of fucose, as shown by the 89% reduction in binding to AAA (Table 1, Fig. 1), a fucose-specific lectin (46). MECA 79 binding to sialidase/fucosidase-treated GlyCAM-1 was reduced 26% relative to its binding to sialidase-treated GlyCAM-1 (Table 1, Fig. 1). In an independent experiment, a reduction of 35% was observed.

The possible importance of sulfate to the MECA 79 epitope was investigated using chlorate as a metabolic inhibitor of sulfation. We previously devised conditions that suppress the sulfation of GlyCAM-1 and other macromolecules within lymph nodes by ~90% without affecting the overall sialylation or fucosylation of GlyCAM-1 or altering the pattern of protein synthesis within the nodes (34). As shown in Fig. 2, undersulfated [^{3}H]galactose-labeled GlyCAM-1 showed undiminished binding to CAMO2 or to *Limax flavus* agglutinin. The decreased mobility of undersulfated GlyCAM-1 on SDS-PAGE may reflect the strong contribution of sulfation to the overall charge of the glycoprotein. In correspondence with previous results obtained with other metabolic

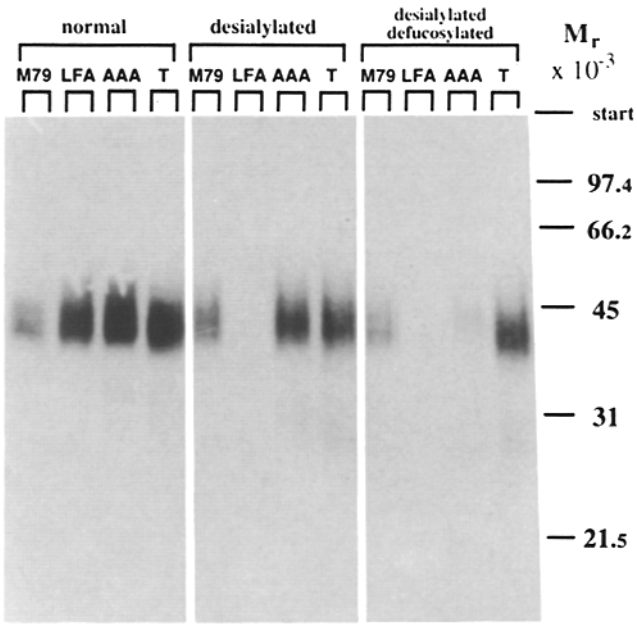

Figure 1. Effects of sialidase and sialidase/fucosidase treatments of GlyCAM-1 on binding to MECA 79. GlyCAM-1, labeled with [^{3}H]galactose and isolated with CAM02 antibody, was either not treated (normal), digested with *Arthrobacter* sialidase (desialylated), or digested with combined *Arthrobacter* sialidase and *Streptomyces* α(1→3/4) fucosidase (desialylated/defucosylated). Each GlyCAM-1 preparation was divided into four equal aliquots, of which three were subjected to precipitation with AAA, a lectin with specificity for fucose moieties; *Limax flavus* agglutinin (LFA), a lectin with specificity for sialic acid moieties, and MECA 79 (M79). The precipitates and the fourth aliquot of each preparation containing the total CAM02-reactive GlyCAM-1 (T) were run on a 10% SDS gel. The gel was analyzed by autoradiography.

Table 1. *Binding of MECA 79 mAb to GlyCAM-1 after Treatments with Sialidase and Fucosidase*

Fraction	Untreated		Sialidase		Sialidase/ α(1→3/4) Fucosidase	
	Amount	Percent total	Amount	Percent total	Amount	Percent total
MECA 79-bound	2194	30	2695	52	1719	38
Limax-bound	5491	74	0	0	3	0
AAA-bound	7096	96	5095	99	501	11
Total input (T)	7363	100	5150	100	4569	100

The data presented in the table were obtained by densitometry (Molecular Dynamics, Sunnyvale, CA) of the autoradiograph depicted in Fig. 1. In this analysis, the region of the autoradiograph limited at the upper end by the 66-kD marker and at the lower end by the 31-kD marker was divided into twelve parallel segments containing the GlyCAM-1 bands of the 12 lanes in the gel. The amount of GlyCAM-1 in each lane (given in arbitrary units) was obtained by integration of the optical density over the entire respective segment with subtraction of the background. Each value is given as a percentage of total GlyCAM-1, with total (T) defined as the amount of CAM02-reactive GlyCAM-1 in the respective sample.

labels (34), the undersulfated GlyCAM-1 exhibited no detectable binding to LEC-IgG. Furthermore, MECA 79 mAb failed to precipitate undersulfated GlyCAM-1. It should be noted that only a subset of the Ab-precipitated GlyCAM-1 was capable of reacting with either LEC-IgG or MECA 79, despite the fact that these reagents were used in great excess. The LEC-IgG reactive subset and, to a lesser degree, the MECA 79-reactive subset corresponded to the higher M_r species of the glycoprotein.

Sulfation dependence was also investigated with [^{3}H]galactose-labeled CD34 isolated from cultured lymph nodes (Fig. 3). Undersulfation did not affect the recognition of CD34 by either the anti-CD34 Ab or *Limax* agglutinin. However, the binding of undersulfated CD34 to LEC-IgG or to MECA 79 was almost completely abrogated. Again, the MECA 79 and LEC-IgG reactive species occurred in a higher M_r subset of the total CD34, as defined by precipitation with the polyclonal CD34 Ab.

To directly compare components from lymph node that were reactive with MECA 79 and LEC-IgG, a detergent lysate

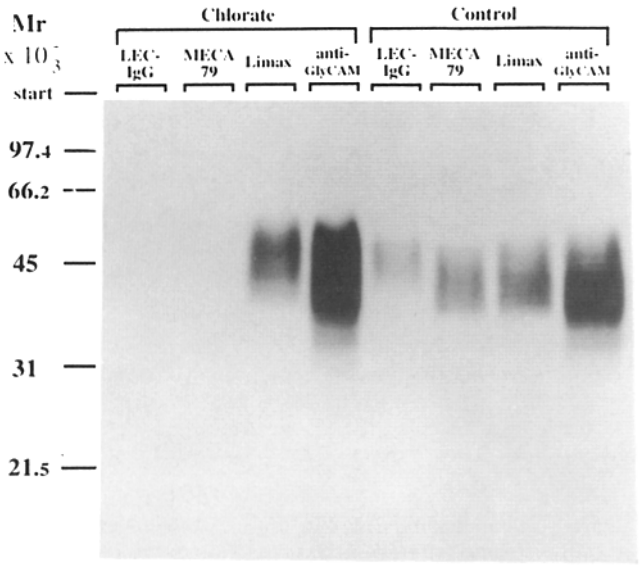

Figure 2. Sulfate dependency of LEC-IgG and MECA 79 interaction with GlyCAM-1. Lymph nodes were labeled with [^{3}H]galactose in the presence or absence of sodium chlorate, and GlyCAM-1 was isolated with CAM02 antibody from a detergent lysate. Antigen from each culture was released from the antibody-beads by specific peptide elution and divided into four equal aliquots. One aliquot (anti-GlyCAM-1) was not subjected to further precipitation while the other three aliquots were precipitated with beads conjugated to *Limax* agglutinin, MECA 79, or LEC-IgG. The four samples from each culture condition were electrophoresed on a 10% SDS gel and the gel was analyzed by autoradiography.

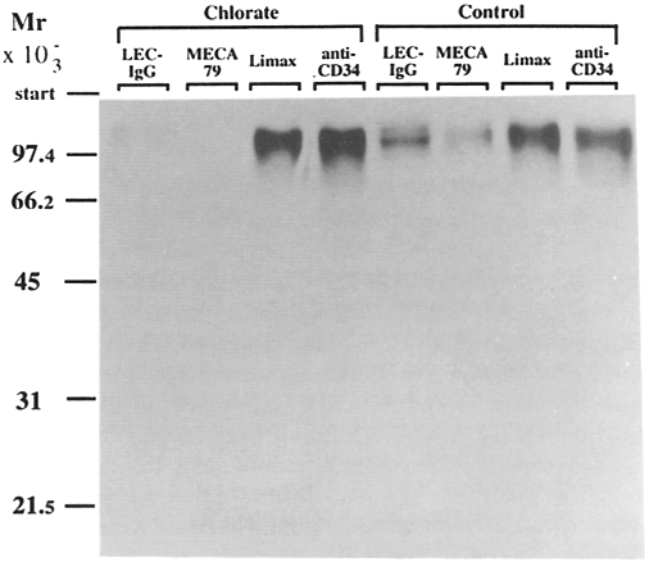

Figure 3. Sulfate dependency of LEC-IgG and MECA 79 interaction with CD34/Sgp90. CD34/Sgp90 was isolated with CD34 antibody from detergent lysates of lymph node cultures, labeled with [^{3}H]galactose in the presence or absence of sodium chlorate. Antigen from each culture was released from the antibody beads by boiling and divided into four equal aliquots. One aliquot (anti-CD34) was not subjected to further precipitation while the other three aliquots were precipitated with beads conjugated with *Limax* agglutinin, MECA 79, or LEC-IgG. The four samples from each culture condition were electrophoresed on a 10% SDS gel and the gel was analyzed by autoradiography.

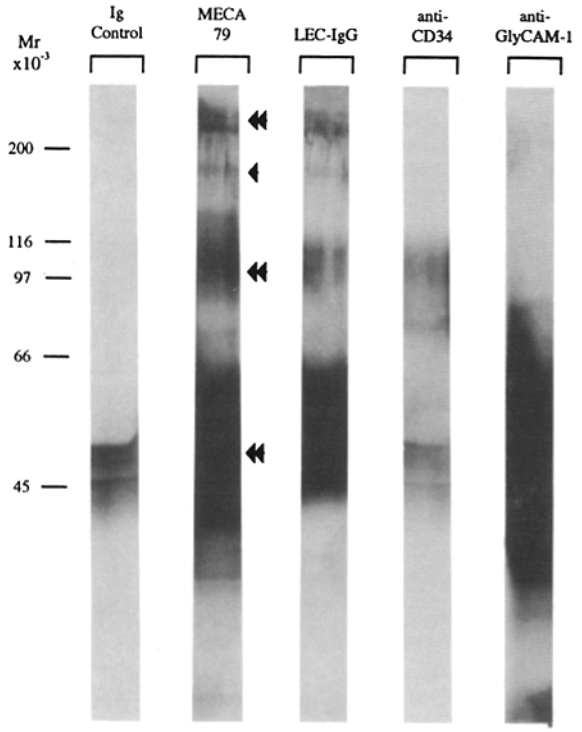

Figure 4. Parallel precipitations from lymph node lysate with MECA 79 and LEC-IgG. Lymph nodes (peripheral and mesenteric) were labeled in organ culture with [^{35}S]SO_4. Equal aliquots of the detergent lysate were precipitated with beads conjugated to Ig control (a mixture of CD4-IgG and rat IgM), MECA 79, LEC-IgG, anti-CD34, or anti-GlyCAM-1 (CAM02). The precipitates were electrophoresed on a 7.5% SDS gel and the gel was analyzed by autoradiography. The *double arrowsheads* denote the bands corresponding to Sgp200, CD34/Sgp90, and GlyCAM-1/Sgp50 whereas the *single arrowhead* denotes the ~170-kD component. In the LEC-IgG precipitate, the CD34 component is somewhat compressed due to the presence of unlabeled LEC-IgG at ~90 kD.

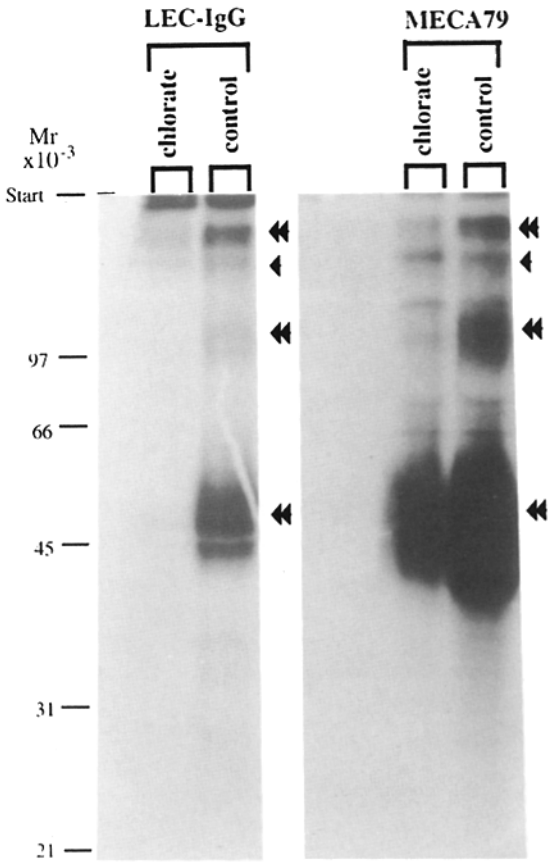

Figure 5. Sulfate dependency of LEC-IgG and MECA 79 interaction with Sgp200. Lymph nodes (peripheral and mesenteric) were labeled in organ culture with [^{3}H]galactose in the presence or absence of chlorate. The detergent lysates from control and chlorate-treated cultures were divided into equal aliquots and precipitated with MECA 79 or LEC-IgG. The precipitates were electrophoresed on a 10% SDS gel and the gel was analyzed by autoradiography. The *double arrowheads* indicate the bands corresponding to Sgp200, CD34, and GlyCAM-1, all of which show sulfate-dependency in their binding to LEC-IgG and MECA 79. The *single arrowhead* denotes the ~170-kD component.

of [^{35}S]SO_4-labeled lymph nodes was subjected to precipitation with the two reagents. Both MECA 79 and LEC-IgG precipitated three major bands at ~50, ~90, and ~200 kD (Fig. 4). The first two components comigrated with GlyCAM-1 and CD34/Sgp90, as shown by precipitation with monospecific polyclonal Abs. The ~200-kD component was apparently distinct, since it was not precipitated by either CAM02 or CD34 Ab (Fig. 4). A relatively minor band at ~170 kD was also precipitated by both LEC-IgG and MECA 79 but not by CAM02 or CD34 Ab.

To examine the sulfate dependency of precipitation, lymph nodes were labeled in organ culture with [^{3}H]galactose in the presence or absence of chlorate. Detergent lysates from these cultures were reacted in parallel with LEC-IgG and MECA 79. From control lymph nodes, each precipitated the same four bands, corresponding to CD34, GlyCAM-1, and the ~200 and ~170 kD components (Fig. 5). Culture of lymph nodes in chlorate dramatically reduced or eliminated the ability of both LEC-IgG and MECA 79 to recognize GlyCAM-1, CD34, and the ~200-kD component (Fig. 5). In contrast, the recognition of the ~170-kD component from chlorate-treated cultures was largely preserved. Besides the four components that were precipitated in common by the two reagents, all of which were relatively diffuse in character, a number of sharp bands were precipitated by MECA 79 (Fig. 5). These bands were judged to be nonspecific components, since they were not reproducibly present in repeat experiments.

Discussion

Our analysis revealed that several [^{35}S]SO_4 and [^{3}H]galactose-labeled glycoproteins within lymph node lysates were precipitated in common by MECA 79 and LEC-IgG i.e., GlyCAM-1, CD34, ~200 kD, and an additional component at ~170 kD. Previous analyses of MECA 79–reactive components, employing radioiodination of immunoprecipitated material, detected bands corresponding in molecular weight to these four components as well as species at 115, 75, and ~60 kD (42).

The ~60-kD molecule is of particular interest, because it constitutes a subset of MAdCAM-1, a novel glycoprotein possessing both Ig-like domains and a mucin domain (42,

47). MAdCAM-1 was originally identified on HEV of mouse Peyer's patches, but it is also present on HEV of mesenteric lymph nodes and peripheral lymph nodes of young mice (42, 48). MAdCAM-1 on Peyer's patch HEV is a ligand for $\alpha_4\beta_7$, a Peyer's patch homing receptor on lymphocytes (49). This adhesive function involves its NH_2-terminal Ig-like domain (Briskin, M., and E. C. Butcher, manuscript in preparation). It is important to note that a MECA 79-reactive subset of MAdCAM-1 on HEV of lymph nodes is recognized by L-selectin and can support L-selectin–dependent lymphocyte rolling (42). Using MECA 79 and LEC-IgG, we were unable to detect an obvious ~60 kD component with either [^{3}H]galactose or [^{35}S]SO_4 as the metabolic label. The dominance of GlyCAM-1 in the 50–60 kD range may have obscured a weak MAdCAM-1 band. In fact, in separate studies using MECA 367 mAb (47) to precipitate MAdCAM-1 from [^{35}S]SO_4-labeled mesenteric lymph nodes, we confirmed that MAdCAM-1 incorporated label, although weakly (Veals, S., and S. Hemmerich, unpublished observations). The limited detectability of this component with MECA 79 and LEC-IgG in our metabolic radiolabeling studies, as opposed to its ready detectability in radioiodination and Western blotting analyses (42), could be due to a low content of sulfate and galactose and/or a slow metabolic turnover. Similar considerations can be invoked to explain the absence of the 75- and 115-kD components in our analysis.

Our experiments demonstrate that LEC-IgG and MECA 79 both react with a major lymph node component at ~200 kD. Because of the evidence implicating this component as an independent HEV-associated ligand for L-selectin (summarized below), we designate it as Sgp200. A similar component was identified in the previous analyses carried out with MECA 79 (42). Also, a molecule of ~190 kD was detected in rat lymph nodes with a rat L-selectin/Ig chimera (50) and a ~250 kD component was detected in human tonsilar HEV with a antibody directed to a complex form of sialyl Lewis X (51). Sgp200 closely corresponds in behavior to GlyCAM-1 and CD34. Desialylation of Sgp200 severely reduced its interaction with LEC-IgG, while allowing its binding to MECA 79 (data not shown). However, the binding of Sgp200 to both LEC-IgG and MECA 79 was dependent on sulfation. Sgp200 did not appear to be an aggregate of either GlyCAM-1 or CD34, since antibodies against these ligands failed to precipitate it. Because the LEC-IgG staining of lymph nodes is largely restricted to HEV (13), we expect that Sgp200, like GlyCAM-1 and the L-selectin reactive form of CD34, will also be localized to HEV.

It is noteworthy that mucin domains are present within the three molecularly characterized glycoproteins (i.e, GlyCAM-1, CD34, and MAdCAM-1) that can function as ligands for L-selectin. Additionally, a recently described myeloid ligand for P-selectin has a large mucin-like domain (52). It seems quite plausible that the multivalent presentation of carbohydrate chains, inherent in this structural organization, is an important feature in defining the avidity of these ligands. We therefore, predict that Sgp200 will also be mucin-like. A major challenge for the future will be to determine the physiologic roles of the different L-selectin ligands.

The distribution of the MECA 79 epitope on multiple components has suggested that it represents a posttranslational modification (41). Since the antibody functionally blocks ligand sites for L-selectin, the most obvious possibility was that the epitope consists of a carbohydrate or modification of a carbohydrate. We have shown here that the sialylation of GlyCAM-1 and CD34 is not required for MECA 79 recognition, although it is essential for the avid interaction of each with L-selectin. In contrast to sialic acid, fucose appears to contribute positively to the MECA 79 epitope. We observed a ~30% reduction of MECA 79 binding to GlyCAM-1 following ~70% removal of fucose. It remains to be seen whether more complete defucosylation would result in a complete loss of the epitope. The most striking finding of the present study is that sulfation is critical for the MECA 79 epitope. Undersulfated GlyCAM-1, CD34, and Sgp200, created by the use of chlorate, were not recognized by MECA 79. Furthermore, as previously shown for GlyCAM-1 and now extended to CD34 and Sgp200, ligand activity for L-selectin was dramatically reduced by undersulfation.

The exact nature of the MECA 79 epitope remains to be defined. In our previous work, we identified the major sulfated mono- and disaccharides of GlyCAM-1 as Gal-6-SO_4, GlcNAc-6-SO_4, (SO_4-6)Galβ1→4GlcNAc and Galβ1→4 (SO_4-6)GlcNAc (36). Furthermore, we showed (37) that a major capping group of the GlyCAM-1 O-linked chains is 6′-sulfated, sialyl Lewis X, i.e., Siaα2→3(SO_4-6)Galβ1→4 (Fucα1→3)GlcNAc. The finding that MECA 79 reacts only with a subset of GlyCAM-1 and CD34, as described also for MAdCAM-1 (42), suggests that the epitope for this mAb may be complex. Future work will be directed at defining this epitope in terms of the sulfated and fucosylated constituents that are present within the O-linked chains of GlyCAM-1.

MECA 79 blocks L-selectin dependent lymphocyte binding to HEV in mouse lymph nodes and in human tonsils and lymph nodes (38, 40). Thus, the sulfation requirement for recognition of PNAd by this antibody, reinforces the previous demonstration that sulfation is essential for HEV-ligand activity (34). It is important to note that MECA 79 staining is induced on venular endothelium in many chronic inflammatory settings, including multiple cutaneous lesions, rheumatoid synovium, lymphocytic thyroiditis, and inflammatory bowel diseases in human (40, 53); a DTH site in sheep (54); inflamed pancreatic acinar tissue in a IL-10 transgenic mouse model (55); inflamed pancreas in a IFN-γ transgenic mouse model (56); and spontaneously inflamed pancreatic islets and salivary glands in the nonobese diabetic (NOD) mouse (57, 58). In the case of NOD mice, expression of the MECA 79 epitope was shown to be associated with the expression of functional ligands for L-selectin (57). It is also noteworthy that strong metabolic incorporation of [^{35}S]SO_4 has been seen, not only in HEV of rodent and human lymph nodes (27, 59), but also in HEV-like vessels that are induced at sites of chronic inflammation in both rodent and human (59, 60). Taken together, these findings raise that the possibility that sulfation, in addition to being essential for the function of HEV-ligands in secondary lymphoid organs, is also required for recognition of L-selectin ligands on blood vessels at sites of chronic inflammation.

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